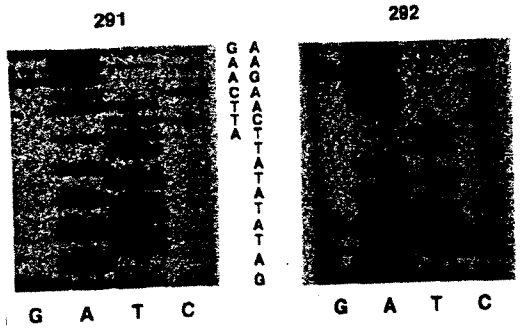

FIG. 3 Direct sequencing of the parents of the affected individual in family BOS22. PCR-amplified DNA was purified and asymmetrically amplified. The resulting single-stranded DNA was sequenced with the CF-17 primer. The sequence for the mother (no. 291) matches that of the father (no. 292) and the published sequence up to the T residue (position 2,566). Beyond this point, the sequence is a mixture of the two alleles owing to an AT insertion. METHODS. DNA amplified with primers CF-8 and CF-17 was electrophoresed on a nondenaturing polyacrylamide gel. The product was excised from the gel and soaked in 100 μl water for 1 h at 65 °C. Eluted DNA (5 μl) was reamplified with a 50-fold dilution of primer CF-17 for 40 cycles. The amplified DNA was purified with a Centricon 100 column (Amicon) and sequenced with Sequenase (USB) using dITP and ^{35}S-labelled dATP.

whereas the other carrier sibling had borderline values at 6–7 years of age (49–50 milliequivalents Cl^-) that decreased to normal levels (38 milliequivalents Cl^-) at 8 years of age. The mother had a normal sweat-test value. The difference in sweat-test values among these individuals could be due to environmental factors or innaccuracies in the sweat test, or reflect additional genetic control over ion transport in the sweat duct. In either case, a more detailed characterization of ion transport and regulation in these individuals should provide insight into these processes.

The CFIns2566 allele is due to the addition of an AT dinucleotide into a short segment (8 bp) of AT dinucleotides. Dinucleotide repeats are hotspots for mutations[19,20]. Although principally CA repeats have been examined, polymorphic AT repeats have also been characterized. A search of the primate sequences in the computerized database GenBank revealed >50 sequences containing AT repeats that were of 22 bp or more (M.D., data not shown). The mechanism for generating new alleles at these loci is not understood, but could involve unequal crossing-over or errors in replication. Examination of new alleles at other tandemly repeated loci, however, indicates that more complex mechanisms could be involved[21].

The identification of all of the mutations that cause CF is essential for complete detection and diagnosis of the disease. Although the CFIns2566 allele seems to be rare, the identification of this mutation provides some important insights. First, all of the CF mutations do not lie in the same exon, implying that complete detection will probably require examination of several regions of the gene. Second, frame-shift and other null mutations might not be uncommon. The most likely explanation for the failure so far to find such mutations in the CF gene is that individuals homozygous for the loss of gene function do not survive. If carriers for termination mutants are healthy, there would be no selection against such alleles; these alleles would only appear in CF individuals, however, when balanced by a less severe allele. Frame-shift mutations could occur in virtually any region of the gene, making CF diagnosis difficult.

The continued identification of mutations in the CF locus is expected to help elucidate which regions of the CFTR are functionally important. Also, examination of the effects of these mutations in the allelic combinations in which they naturally occur should greatly increase our understanding of the function of the CFTR gene and its role in disease. □

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Biological properties of a CD4 immunoadhesin

Randal A. Byrn*, Joyce Mordenti, Catherine Lucas, Douglas Smith, Scot A. Marsters, Jennifer S. Johnson*, Paul Cossum, Steven M. Chamow, Florian M. Wurm, Timothy Gregory, Jerome E. Groopman* & Daniel J. Capon

Genentech Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080, USA
* Division of Hematology-Oncology, Harvard Medical School, New England Deaconess Hospital, Boston, Massachusetts 02215, USA

MOLECULAR fusions of CD4, the receptor for human immunodeficiency virus (HIV; refs 1–4), with immunoglobulin (termed CD4 immunoadhesins) possess both the gp120-binding and HIV-blocking properties of recombinant soluble CD4, and certain properties of IgG, notably long plasma half-life and Fc receptor binding[5,6]. Here we show that a CD4 immunoadhesin can mediate antibody-dependent cell-mediated cytotoxicity (ADCC) towards HIV-infected cells, although, unlike natural anti-gp120 antibodies, it does not allow ADCC towards uninfected CD4-expressing cells that have bound soluble gp120 to the CD4 on their surface. In addition, CD4 immunoadhesin, like natural IgG molecules, is efficiently transferred across the placenta of a primate. These observations have implications for the therapeutic application of CD4 immunoadhesins, particularly in the area of perinatal transmission of HIV infection.

We have previously described CD4 immunoadhesins containing the first two immunoglobulin-like domains of CD4 joined to the entire constant region of human IgG1 heavy chain[5]. As the presence of light chain was found to be unnecessary for secretion of dimeric molecules[5], we constructed additional derivatives lacking the CH1 domain of the IgG1 heavy chain (Fig. 1). The gp120-binding and Fc receptor-binding properties and the improved half-life characteristics of this molecule were comparable to the CD4 immunoadhesin containing the CH1 domain (not shown).

As CD4 immunoadhesin binds Fc receptors, we examined

TABLE 1 Placental transfer of rCD4 and CD4-IgG in pregnant rhesus monkeys

Rhesus monkey	Protein	Concentration (ng ml^{-1}) in maternal serum		Concentration (ng ml^{-1}) in newborn serum	Infant/maternal concentration ratio
		Mean	Range		
1	CD4-IgG	489	(276–673)	15.2	3.1%
2	CD4-IgG	217	(155–301)	7.6	3.5%
3	rCD4	682	(360–820)	1.1	0.16%
4	rCD4	437	(205–504)	<0.8	<0.18%

Four pregnant rhesus monkeys at 150–160 days gestation (normal gestation period 160–170 days) received a loading dose of CD4 immunoadhesin (CD4-IgG) or rCD4 by rapid intravenous injection followed by continuous infusion for 24 h; the infants were delivered by caesarian section. Blood was obtained from the mother after 1 min and after 4, 8, 12, 16, 20 and 24 h of infusion and from the infant and cord blood at the time of delivery. For maternal dosing and blood sampling, catheters were placed into a femoral vein and artery under general anaesthesia 24 h before the start of the study. After catherization the animals were placed into jackets, and no additional anaesthesia was given. Animals received the loading dose of drug as an intravenous bolus into the femoral vein catheter over 5 s followed by saline flush to clear the catheter of drug. The infusion was started immediately thereafter. For CD4 immunoadhesin, a 0.135 mg kg^{-1} loading dose was given, followed by 1.12 mg kg^{-1} over 24 h; for rCD4, a 0.135 mg kg^{-1} loading dose was given, followed by 28 mg kg^{-1} over 24 h. Serum concentrations of each protein were determined by double antibody enzyme-linked immunosorbent assays (ELISA) each using monoclonal antibody Leu3a (Becton–Dickinson). As this antibody recognizes the gp120 binding domain of CD4, the assays thus detect CD4-containing molecules still capable of binding gp120. To measure rCD4 concentration, Leu3a in 0.05 M carbonate buffer, pH 9.6, was used to coat 96-well microtitre plates overnight at 4 °C. After three washes with PBS containing 0.05% Tween 20 (PBS-Tween), plates were blocked for 1 h at room temperature with ELISA diluent (PBS containing 0.5% BSA, 0.05% Tween 20 and 0.01% thimerosal). rCD4 standards and samples diluted in rhesus serum were incubated for 2 h, and plates were washed again with PBS-Tween. For detection of rCD4, monoclonal antibody OKT4 (Ortho) was conjugated to horseradish peroxidase (HRP, Boehringer Mannheim) by the periodate method[19]. After appropriate dilution in ELISA diluent, the conjugated antibody was incubated for 1 h at ambient temperature. Orthophenylene diamine dihydrochloride (Sigma), 2.2 mM in 0.05 M sodium phosphate/0.1 M citrate buffer, pH 5.0, containing 0.01% H_2O_2, was used as a substrate for 20–30 min at room temperature. Reactions were stopped with 4.5 N H_2SO_4 and plates were read at 492 nm. Data were reduced using a four-parameter curve-fitting program[20]. The range for this assay was 0.8 to 25 ng ml^{-1}. For the measurement of CD4 immunoadhesin, the same procedure was used, except that Leu3A was conjugated to HRP and used for detection; monoclonal antibody L104.5 (provided by B. Fendly, Genentech), which recognizes domain 2 of rCD4, was used for antigen capture. The range for this assay was 0.19 to 12.0 ng ml^{-1}.

whether it could mediate ADCC towards HIV-infected cells by human peripheral blood mononuclear cells. Indeed, CD4 immunoadhesin mediates ADCC towards HIV-infected, but not uninfected, CEM human T-lymphoblastoid cells in a dose-dependent manner (Fig. 2*a* and *b*). Soluble recombinant (rCD4) does not mediate ADCC (not shown), but can inhibit cell lysis mediated by CD4 immunoadhesin (Fig. 2*a*), demonstrating that specific binding to gp120 by CD4 immunoadhesin is essential.

It has been suggested that ADCC in AIDS patients may be a mechanism of pathogenesis rather than protection[7], as soluble gp120, by binding to healthy CD4-expressing 'bystander' cells, can make such cells targets for ADCC, mediated by the anti-gp120 antibodies found in HIV-infected individuals. In contrast to natural anti-gp120 antibodies, CD4 immunoadhesin does not mediate killing of uninfected CEM cells preincubated with soluble gp120 (Fig. 2*b*). A likely explanation is that CD4 immunoadhesin, unlike natural anti-gp120 antibodies, cannot bind gp120 already bound to cell-surface CD4, because soluble gp120 is thought to have only one CD4-binding site.

An increasing number of paediatric AIDS patients are infected *in utero* by transmission from the mother[8]. As natural IgG molecules are selectively transported across the placenta of primates in an Fc receptor-dependent manner, we tested whether CD4 immunoadhesin shared this property. Pregnant rhesus monkeys near to term were given a bolus dose of either rCD4 or CD4 immunoadhesin, then were continuously infused to a relatively constant concentration for 24 h before delivery by caesarian section. Serum concentrations were determined by

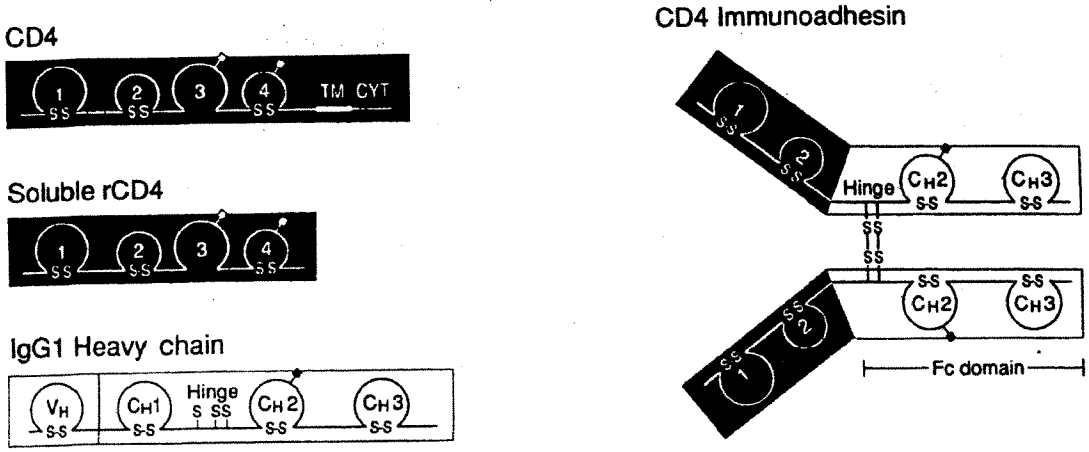

FIG. 1 Structure of CD4 immunoadhesin, soluble rCD4 and the parent human CD4 and IgG1 heavy chain molecules. CD4- and IgG1-derived sequences are indicated by shaded and unshaded regions, respectively. The immunoglobulin-like domains of CD4 are numbered 1–4; TM and CYT refer to the transmembrane and cytoplasmic domains. Soluble rCD4 is truncated after proline 368 of the mature CD4 polypeptide[14]. The variable (VH) and constant (CH1, hinge, CH2, and CH3) regions of IgG1 heavy chain are shown. Disulphide bonds are indicated by S–S. CD4 immunoadhesin consists of residues 1–180 of the mature CD4 protein fused to IgG1 sequences beginning at aspartic acid 216 (taking amino acid 114 as the first residue of the heavy chain constant region[15]) which is the first residue in the IgG1 hinge after the cysteine residue involved in heavy-light chain bonding. The CD4 immunoadhesin shown, which lacks a CH1 domain, was derived from a CH1-containing CD4 immunoadhesin[5] by oligonucleotide-directed deletional mutagenesis[16], expressed in Chinese hamster ovary cells and purified to >99% purity using protein A-Sepharose chromatography as described[5].

immunoassay at various times in the mother and in the newborn within 5 min of birth. The concentration of CD4 immunoadhesin in fetal serum was ⩾3% of the maternal level after 24 h (Table 1), indicating a significant rate of placental transfer. By contrast, rCD4 did not accumulate in the fetal serum to a significant extent. This is most probably due to lack of active transport across the placental barrier, although it is possible that transfer would not be detected owing to the shorter half-life of rCD4 (ref. 5).

Although the rate at which a protein appears in the fetal circulation cannot be directly translated into a rate of placental transfer, because the rate of degradation of the protein in the fetus is unknown, comparisons can be made with the appearance rate of human antibody in classical human experiments. Dancis *et al.*[9] gave radioiodinated human γ-globulin to women in their third month of pregnancy before abortion of the fetus, and observed a concentration in the fetus that was 2.8% of maternal levels after 18–24 h. Simiarly, Gitlin *et al.*[10] gave women who were nearly to term a single intravenous injection of radioiodinated γ-globulin up to 4 weeks before birth and observed an increase in the infant plasma concentration of ~3% of maternal level per day. Thus the rate of appearance of CD4 immunoadhesin in a primate fetus is close to that of normal human IgG in humans.

CD4-based strategies have an important theoretical advantage over other AIDS therapeutics, as HIV must bind CD4 to be able to infect its cellular target (the T4 cell) specifically. Soluble CD4 derivatives have thus been developed with two objectives: to block gp120-mediated events such as the spread of viral infection, formation of syncytia and binding of gp120 to uninfected 'bystander' cells, and to use CD4 as a targeting agent to direct a cytotoxic agent to HIV-infected cells (for example, CD4-ricin[11], CD4-pseudomonas exotoxin[12]). Here we have shown that CD4 immunoadhesin can direct the killing of HIV-infected cells, as well as blocking gp120-mediated events[5,6]. Significantly, CD4 immunoadhesin, unlike natural anti-gp120 antibodies, cannot kill CD4-expressing bystander cells coated with soluble gp120.

The fetal acquisition of passive immunity in humans is mediated by selective placental transfer of maternal IgG. As CD4 immunoadhesin shares this property, passive immunity to HIV could be established in the fetus by maternal administration, possibly preventing perinatal transmission of infection. The mechanism underlying selective transport of IgG involves binding to Fc receptors on the apical surface of the syncytiotrophoblast, resulting in protected endocytotic transport[13]. The fact that this, and so many other different properties of IgG, can be conferred on CD4 by the addition of an Fc region suggests that such functions could be acquired by any adhesion molecule capable of being linked to Fc in place of the Fab sequences

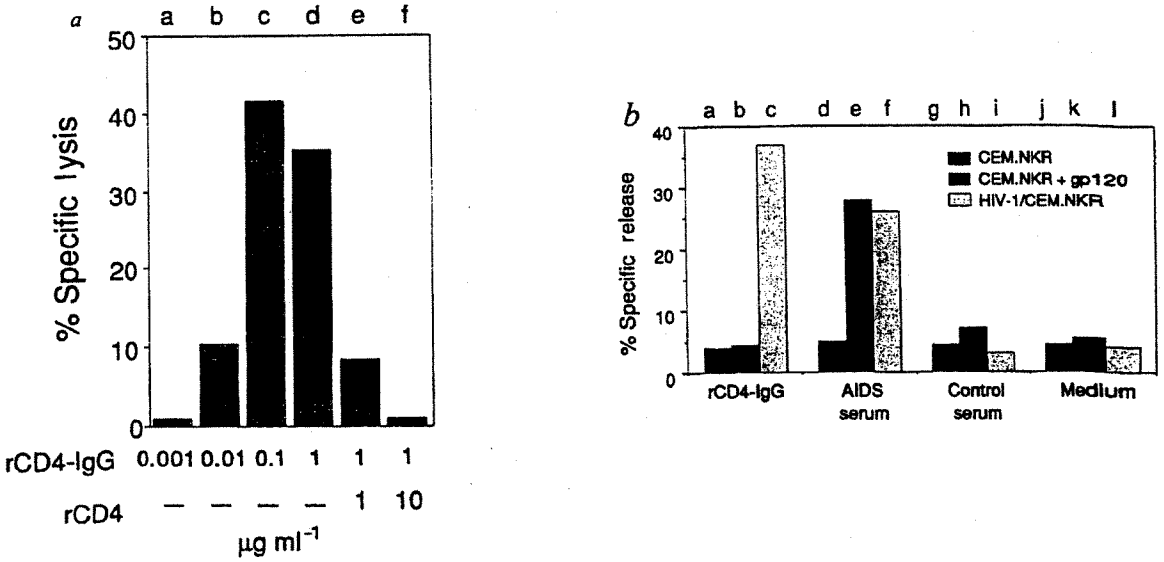

FIG. 2 Antibody-dependent cell-mediated cytotoxicity (ADCC) shown by CD4 immunoadhesin. CEM T-lymphoblastoid target cells were labelled with ^{51}Cr, incubated with CD4 immunoadhesin, rCD4, serum, or control media for 30 min, and mixed with peripheral blood mononuclear cells (PBMC), as effector cells, at an effector-to-target ratio of 50:1. The cell mixtures were incubated for 20 h at 37 °C, and the cell-free supernatant was collected and assayed for ^{51}Cr released from target cells. *a*, Lysis of HIV-1-infected CEM.NKR target cells by effector cells in the presence of CD4 immunoadhesin at 0.001 (lane a), 0.01 (lane b), 0.1 (lane c) and 1.0 μg ml^{-1} (lanes d–f). Also shown is the blocking by rCD4 at 1.0 (lane e) and 10 μg ml^{-1} (lane f) of target cell lysis mediated by 1.0 μg ml^{-1} CD4 immunoadhesin. The level of cell lysis observed with CD4 immunoadhesin was comparable to that mediated by a control AIDS patient serum. rCD4 itself does not mediate target cell lysis at concentrations up to 10 μg ml^{-1}. Uninfected CEM.NKR targets were not lysed by effector cells in the presence of CD4 immunoadhesin, AIDS patient serum or normal human serum (see below), but could be lysed in the presence of a rabbit anti-rCD4 serum (not shown). *b*, ADCC towards uninfected CEM.NKR target cells (lanes a, d, g and j), uninfected CEM.NKR cells incubated with soluble rgp120 (ref. 17) (lanes b, e, h and k) and HIV-1-infected CEM.NKR (lanes c, f, i and l) mediated by CD4 immunoadhesin (lanes a–c), AIDS patient serum at 1/1,000 final dilution (lanes d–f), serum from an uninfected individual at 1/1,000 final dilution (g–l) and complete medium (lanes j–l).

METHODS. The CEM.NKR T-lymphoblastoid cell line, which is resistant to NK-mediated lysis[18], was used for all experiments. HIV-1-infected CEM.NKR cells were produced by inoculating 10^6 CEM.NKR cells with 10^3 $TCID_{50}$ of HIV-1 III_B. The culture was monitored for infection using reverse transcriptase (RT) activity and HIV-1 specific antibodies for immunofluorescence. After ~2 weeks the culture became stable, with >70% of cells immunofluorescence-positive, and >10^6 c.p.m. ml^{-1} RT activity in the medium. Cells were maintained in RPMI 1640 medium (Gibco) containing 20% fetal bovine serum (MA bioproducts), penicillin, streptomycin and L-glutamine (complete medium). Target cells were labelled by incubation of 10^6 cells with 100 μCi ^{51}Cr in 0.5 ml for 2 h at 37 °C. After two washes, cells were suspended at 2×10^5 per ml in complete medium and 25-μl aliquots (containing 5×10^3 cells) were dispensed to wells of a 96-well plate. For the lysis assay, 25 μl purified recombinant proteins or sera diluted in complete medium, or control medium, were added to each well and incubated for 30 min at room temperature. Assays were carried out in triplicate. Effector PBMCs were prepared from heparinized blood obtained from a healthy, HIV-seronegative donor by centrifugation through Ficoll-Paque (Pharmacia). After two washes in RPMI 1640 the cells were suspended to 5×10^6 cells per ml in complete medium and 50-μl aliquots were added to appropriate wells. The total incubation volume was therefore 100 μl. The concentrations indicated are final concentrations after effector cells were added. Plates were incubated for 16–18 h at 37 °C in 5% CO_2. For analysis of cell lysis, 50-μl samples of supernatant were pipetted from each well, mixed with detergent to inactivate HIV, then mixed with 0.5 ml Protosol (New England Nuclear) and 5 ml Betafluor (National Diagnostics) and analysed by scintillation counting. Maximum lysis, spontaneous lysis and complete medium controls were included in each assay for each target cell, in triplicate. Maximum lysis was obtained by substituting 25 μl of 2% Triton X-100 for the test sample. Spontaneous release wells received 70 μl complete medium instead of effector cells. Complete medium controls received medium instead of the test sample. Percentage specific lysis was calculated using the formula, % specific lysis = (test sample–spontaneous release)/(maximum lysis–spontaneous release).

normally constituting the antigen-binding site of IgG. Therefore, in principle, any such receptor can be given the functional characteristics of an antibody, with the ability to select desirable characteristics at will. □

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Calcium entry through stretch-inactivated ion channels in *mdx* myotubes

Alfredo Franco Jr & Jeffry B. Lansman*

Department of Pharmacology, School of Medicine, University of California, San Francisco, California 94143-0450, USA

RECENT advances in understanding the molecular basis of human X-linked muscular dystrophies (for a review, see ref. 1) have come from the identification of dystrophin, a cytoskeletal protein associated with the surface membrane[2–4]. Although there is little or virtually no dystrophin in affected individuals[5,6], it is not known how this causes muscle degeneration. One possibility is that the membrane of dystrophic muscle is weakened and becomes leaky to Ca^{2+} (refs 7–9). In muscle from *mdx* mice, an animal model of the human disease[10], intracellular Ca^{2+} is elevated and associated with a high rate of protein degradation[11]. The possibility that a lack of dystrophin alters the resting permeability of skeletal muscle to Ca^{2+} prompted us to compare Ca^{2+}-permeable ionic channels in muscle cells from normal and *mdx* mice. We now show that recordings of single-channel activity from *mdx* myotubes are dominated by the presence of Ca^{2+}-permeable mechano-transducing ion channels. Like similar channels in normal skeletal muscle, they are rarely open at rest, but open when the membrane is stretched by applying suction to the electrode[12–14]. Other channels in *mdx* myotubes, however, are often open for extended periods of time at rest and close when suction is applied to the electrode. The results show a novel type of mechano-transducing ion channel in *mdx* myotubes that could provide a pathway for Ca^{2+} to leak into the cell.

We recorded single-channel activity from cell-attached patches on myotubes from normal and *mdx* mice with 110 mM $BaCl_2$ in the patch electrode. Figure 1*a* shows a continuous record of single-channel activity recorded ~1 min after the patch electrode formed a seal on the surface of a myotube from normal mouse muscle. At a holding potential of −60 mV, the single-channel

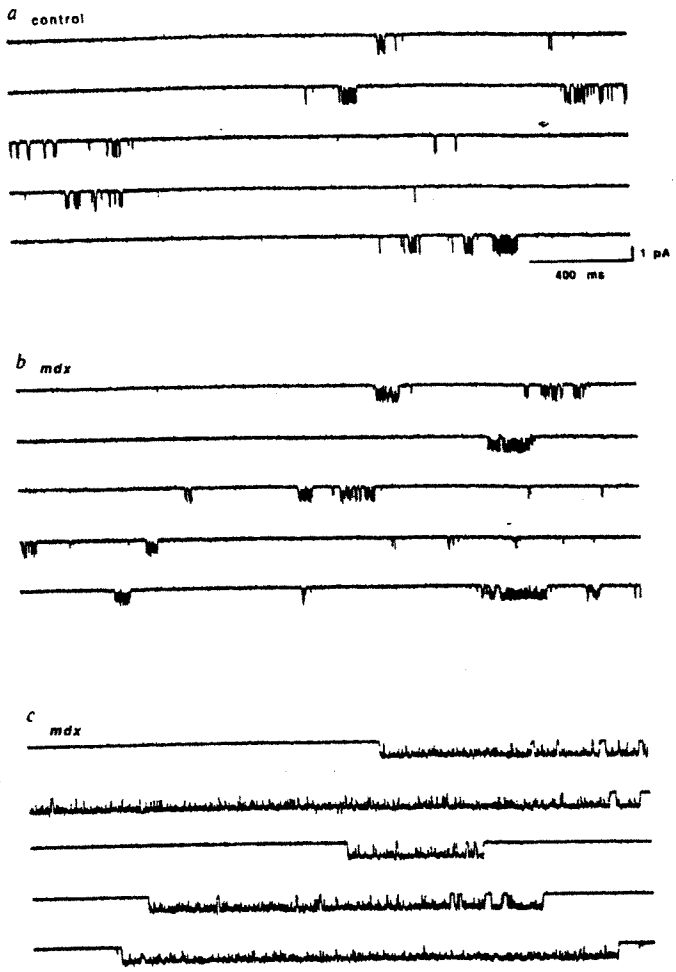

FIG. 1 Channel activity recorded from the surface of myotubes from normal and *mdx* mice with 110 mM $BaCl_2$ in the patch electrode showing unitary Ba^{2+} currents at a constant holding potential of −60 mV. The traces are sequential and represent a segment of a continuous recording (~10 seconds channel activity). Currents were filtered at 1 kHz with an eight-pole Bessel filter and sampled at 5 kHz. *a*, Recording from a cell-attached patch on a normal myotube. *b*, Recording from a cell-attached patch on a *mdx* myotube showing low channel activity. *c*, Recording from a different *mdx* myotube in which channel activity was high.

METHODS. Myotubes were prepared by dissecting hind-limb or cutaneous-pectoris muscles from 7-day-old normal C57B control mice or *mdx* mice (Jackson Laboratory) after killing by cervical dislocation. The muscle was minced and incubated for ~15 min at 37 °C in Ca^{2+}- and Mg^{2+}-free Hank's buffer containing 0.125% typsin. Cells were dissociated by passing through a small-bore pipette and filtered through 100-μm gauze. The suspension was preplated for ~1 h to remove fibroblasts, after which the remaining cells in suspension were plated on gelatin-coated tissue culture dishes at a density of ~5,000 cells per cm^2 in DMEM medium supplemented with 20% FCS and chick embryo extract. Myoblasts began to fuse and form myotubes after ~4–5 days in culture. Recordings were made from myotubes 1–5 days after the first myotubes formed. Recordings of single-channel activity from cell-attached patches were made with a List EPC-7 amplifier as described previously[19]. Current signals were recorded on video tape and replayed onto the hard disk of a laboratory computer (PDP 11/73) for later analysis. Patch electrodes were made from borosilicate capillary pipettes (Rochester Scientific) and had resistances of 2–4 MΩ when filled with 110 mM $BaCl_2$ and immersed in the bath. The bathing solution contained 150 mM potassium asparatate, 5 mM $MgCl_2$, 5 mM EGTA, 10 mM glucose and 10 mM HEPES buffer. The pH was adjusted to 6.5 with KOH. An isotonic potassium bathing solution was used to zero the resting potential of the cell. Occasionally, voltage shifts were detected after patch excision which indicated a maximum voltage error of ~10 mV. The bathing solution produced no obvious signs of cell deterioration.

* To whom correspondence should be addressed.